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A PHSYICAL BASIS FOR DEGENERACY IN THE AMINO ACID CODE

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If two or more nucleotide sequences specify the same amino acid, the code is said to be degenerate for that amino acid. Evidence that such degeneracy exists in *E. coli* comes from experiments on the stimulation of ribosomal incorporation of amino acids by synthetic polyribonucleotides. For instance, Martin *et al.*[1] and Speyer *et al.*[2] found that either poly UC or poly UG stimulates incorporation of leucine, so that at least two different combinations code for the same amino acid. Since the specificity of coding seems to reside in sRNA molecules functioning as adaptors,[3, 4] such degeneracy might be due to the existence of two sRNA acceptors for leucine, each having different coding properties. Two peaks of leucine-acceptor activity were demonstrated in yeast sRNA by Doctor *et al.*[5] using countercurrent distribution. Berg and Lagerkvist[6] have shown that *E. coli* sRNA also contains two leucine acceptors with different acceptor-terminal nucleotide sequences. In this paper, two leucine acceptors in *E. coli*, separated by countercurrent distribution, are shown to have different coding properties. One responds preferentially to poly UC, the other to poly UG.

Materials and Methods.—Separation of two leucine acceptors: sRNA from *E. coli*, strain KB, was prepared by phenol extraction and passage over DEAE cellulose as described by Holley *et al.*,[7] then stripped of amino acids, dialyzed, and lyophilized, as described by von Ehrenstein and Lipmann.[8] Countercurrent distribution was done exactly as described by Apgar *et al.*[9] 200 mg of *E. coli* sRNA was added to the first 5 tubes of a 200-tube apparatus, and the final fractions were combined into 40 sets of 5 tubes each, dialyzed, recovered by evaporation, and each dissolved in 2 ml of deionized water. A 100,000 × *g* supernatant of an *E. coli* extract was used as the source of amino acyl RNA synthetase activity. For assays of acceptor activity of the sRNA fractions, the reaction mixture contained in a volume of 0.5 ml: Tris-HCl, pH 7.2, 50 μM; $MgCl_2$, 5 μM; KCl, 5 μM; adenosine triphosphate, Na salt (ATP), 0.5 μM; 0.2 μc of C^{14}-amino acid, either DL-

leucine, 10 $\mu c/\mu M$, or L-arginine, 5 $\mu c/\mu M$ (Calbiochem); reduced glutathione (GSH), 2 μM; bovine serum albumin (BSA), 100 μg; *E. coli* 100,000 $\times$ *g* supernatant, 750 μg protein, 0.05 ml of the countercurrent fraction to be tested. After incubation for 25 min at 30°C, 3 ml cold salt-ethanol (0.5 *M* NaCl, 67% ethanol) and 1 mg of crude yeast RNA were added and the tubes chilled in an ice bath for 5 min. After 4 centrifugations at 4°C (resuspending in salt-ethanol), the precipitate was dissolved in 1.5 *N* NH_4OH, dried, and assayed for radioactivity using a thin-window, gas-flow counter (Nuclear Chicago Corporation), with a C^{14}-counting efficiency of 19%.

Attachment of C^{14}-leucine to peak I and peak II sRNA: The reaction mixture for attaching C^{14}-leucine to the sRNA fractions contained in a total volume of 1 ml: Tris-HCl, pH 7.2, 100 μM; $MgCl_2$, 10 μM; KCl, 10 μM; GSH, 2 μM; ATP, 2.5 μM; C^{14}-leucine, 138 $\mu c/\mu M$ (New England Nuclear Corporation), 0.024 μM; 19 C^{12}-amino acids (leucine omitted), 0.2 μM each; 100,000 $\times$ *g* supernatant protein, 1.5 mg; sRNA from peak I or peak II (Fig. 1), 0.38 or 0.19 mg, respec-

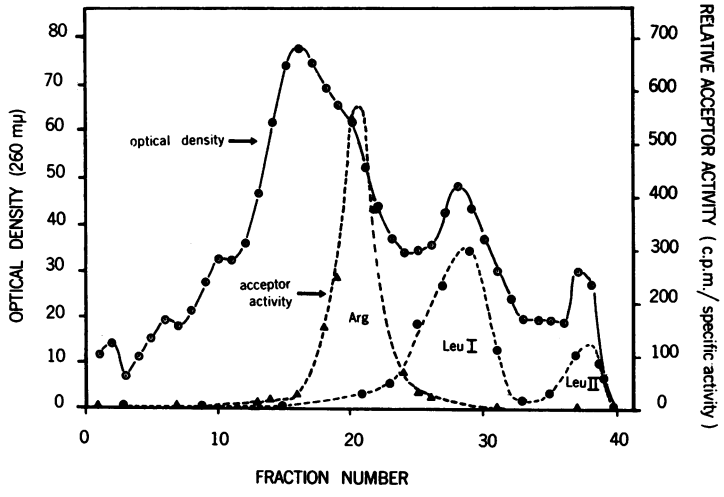

FIG. 1.—Resolution of two leucine acceptors by countercurrent distribution of *E. coli* sRNA. Arginine acceptor activity is shown for comparison. The optical density refers to that of each countercurrent fraction dissolved in 2 ml of water. For the assay of acceptor activity, 0.05 ml was taken from each tube.

tively. After incubation for 30 min at 35°C, the reaction was stopped by addition of 1 ml phenol, and the charged sRNA was isolated as described by von Ehrenstein and Lipmann.[8] The amino acyl-sRNA obtained contained 570,000 cpm per mg sRNA in the case of peak I, and 520,000 cpm per mg sRNA in the case of peak II.

Attachment of C^{14}-leucine to unfractionated sRNA: The reaction mixture was similar to above with the addition of an ATP-generating system. It contained in a total volume of 2 ml: Tris-HCl, pH 7.2, 200 μM; $MgCl_2$, 20 μM; KCl 20 μM; GSH, 4 μM; ATP, 5 μM; phosphoenolpyruvate (PEP), Na salt (Calbiochem), 25 μM; PEP kinase (Boehringer), 40 μg; C^{14}-leucine, 10 $\mu c/\mu M$ (Calbiochem), 0.1 μM; sRNA, 20 mg; 100,000 $\times$ *g* supernatant, 3 mg protein. In this particular preparation, no mixture of C^{12}-amino acids was included. After incubation for 30 min at 35°C, the charged sRNA was isolated by the phenol procedure as above. The product was dialyzed for 18 hr against deionized water at 4°C, and stored frozen at −20°C. It contained 8,600 cpm per mg sRNA.

For attachment of the C^{12}-amino acid mixture to sRNA, the same proportions were used, except that the C^{14}-leucine was replaced by 2 μM each of 19 amino acids (leucine omitted).

Ribosomal preparation: Preincubated 30,000 $\times$ *g* supernatant (iS-30) of *E. coli* extract was prepared as described by Nirenberg and Matthaei,[11] omitting DNase. The preparation used had an OD_{260} of 223 and contained 20 mg protein per ml. When tested for activity as described,[10, 11] 180-fold stimulation of incorporation of free C^{14}-phenylalanine was produced by 10 μg of poly U. Under conditions similar to those used in the leucine transfer experiments below, 75% of sRNA-

bound C^{14}-phenylalanine was found to be incorporated into the hot trichloroacetic acid (TCA)-insoluble product in the presence of 10 μg of poly U, in agreement with Nirenberg *et al.*[11]

Ribosomal incorporation of C^{14}-leucine bound to sRNA: The reaction mixture contained, in a total volume of 1 ml: Tris-HCl, pH 7.6, 100 μM; $MgCl_2$, 14 μM; KCl, 50 μM; GTP, 0.03 μM; PEP, 5 μM; PEP kinase, 20 μg; β-mercaptoethanol, 5 μM; C^{14}-leucyl-sRNA (unfractionated, or from peak I or peak II), as stated in the legends of Figures 2, 3, and 4; C^{12}-amino acyl-sRNA (leucine omitted), 0.1 mg; C^{12}-leucine, 2 μM; 5 μg of either poly U, poly UC, or poly UG, as stated; iS-30, 0.1 ml. Under the conditions used, the amount of C^{14}-leucine incorporated into the acid-insoluble product in 10 min was proportional to the input C^{14}-leucyl-sRNA.

After incubation for the various stated times, the reaction was stopped by addition of 5 ml of 5% TCA and the resulting precipitate washed, digested, and plated as described by Siekevitz.[12]

Synthetic polyribonucleotides: Poly UC (input ratio 1:1) and poly UG (input ratio 5:1) were kindly donated by Dr. S. Ochoa and Dr. R. F. Beers, respectively. Poly U was purchased from the Miles Laboratories, Clifton, New Jersey.

Results.—Separation of two leucine acceptors: Figure 1 shows the results of a countercurrent fractionation of *E. coli* sRNA. The assay of leucine-acceptor activity shows two clearly separated peaks. Samples taken from these will be subsequently referred to as "peak I" and "peak II" sRNA.

Coding properties of peak I and peak II sRNA: Samples of peak I and peak II sRNA were charged with C^{14}-leucine and their response to specific polymers was determined. Nirenberg *et al.*[11] have shown that poly U-stimulated incorporation of C^{14}-phenylalanine can take place by direct transfer of the amino acid from the amino acyl-sRNA complex. The same applies to sRNA-bound cysteine in response to poly UG, as shown by Chapeville *et al.*[4] While the observations that both poly UC and poly UG stimulate ribosomal incorporation of leucine were made using the free amino acid, it can be seen below that the same result applies to leucine bound to sRNA.

Figure 2 shows the kinetics of transfer of C^{14}-leucine from peak I sRNA in response to 3 different polymers. Poly UC produces the highest level of stimulation. A strikingly different result is observed if leucine is transferred from peak II sRNA, as shown in Figure 3. In that case, poly UG is the most active, although poly U also has some effect. It is concluded therefore that peak I sRNA and peak II sRNA have different coding properties.

Note in Figure 3 that peak II responds somewhat to Poly U. It is unlikely that this effect is due to contamination of the poly U with G, since base analysis of the polymer showed that the G content, if any, was less than 0.5%.

Figure 4 gives, for comparison, the results for unfractionated sRNA (not taken through the countercurrent procedure). The pattern of response to the various polymers corresponds roughly to the sum of both peaks. In making the comparison, one must take into account that peak I is about four times as abundant as peak II and also that the input of counts in Figure 2 is about twice that in Figure 3. Thus, there is no indication that the properties of the sRNA were significantly altered by the countercurrent fractionation. A reservation must be noted here, however. In preparing the C^{14}-leucyl-sRNA used in Figure 4, no mixture of C^{12}-amino acids was added. Therefore, it cannot be excluded that minor impurity amino acids in the C^{14}-leucine could have made some contribution to the transfer observed in Figure 4.

Discussion.—Degeneracy, in the case of leucine, can apparently be accounted for by the different coding properties of two acceptors. Berg and Lagerkvist[6]

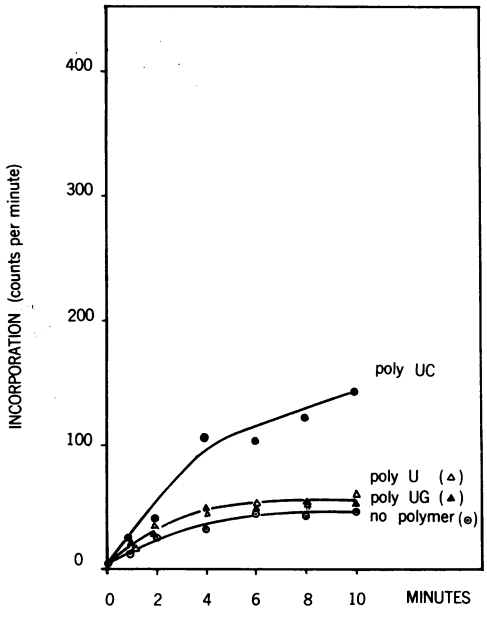

FIG. 2.—Polymer-dependent incorporation of leucine bound to peak I sRNA. The input of C^{14}-leucyl peak I-sRNA was 0.012 mg, containing 6,700 cpm. 0.1 mg C^{12}-amino acyl-sRNA (minus leucine) was also added. The procedure is described in *Methods*.

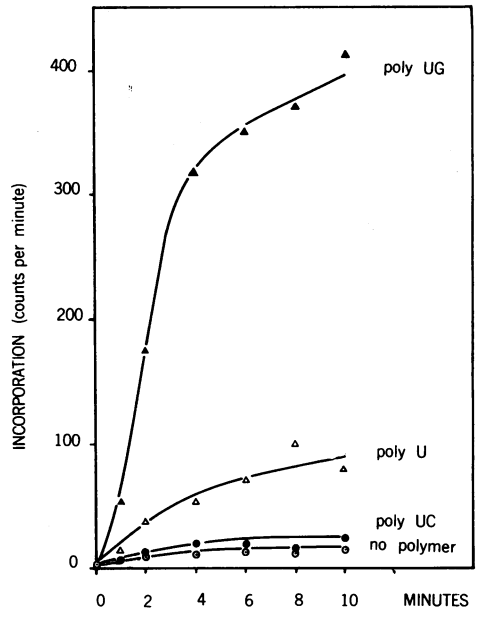

FIG. 3.—Polymer-dependent incorporation of leucine bound to peak II sRNA. The reaction mixture and procedure were as in Fig. 2, except that the labeled input was 0.006 mg of C^{14}-leucyl peak II-sRNA, containing 3,100 cpm.

found two nucleotide sequences at the acceptor terminus for the leucine-acceptor sRNA in *E. coli*—GCACCA and GUACCA in proportion four to one. This relative abundance agrees with the sizes of peak I and peak II. However, there is no obvious complementary relation between these sequences and the responses of peak I and peak II sRNA's to poly UC and poly UG. This suggests that the coding properties of the sRNA molecule are not determined by these residues alone.

Two separable acceptors have also been demonstrated by countercurrent distribution for histidine,[13] threonine, and leucine[5] in yeast, as well as lysine[14] in rat liver. Sueoka and Yamane,[15] using a different technique, have demonstrated multiple peaks in *E. coli* sRNA for various amino acids. However, the existence of two sRNA's for a given amino acid does not necessarily imply degeneracy in coding, since modification of an sRNA molecule may be possible without altering its coding specificity.

Whether degeneracy is widespread in nature and plays an important part in protein synthesis remains to be determined. Some degree of degeneracy may confer increased survival value upon a cell, by reducing the number of nonsense coding units, i.e., those that correspond to no amino acid. A mutation that changes one amino acid in a polypeptide chain to another amino acid is less likely to be deleterious than one which completely impedes the completion of the growing chain.[16]

It should be pointed out that two kinds of degeneracy are possible. The first kind is illustrated by the difference between peak I and peak II, a given amino acid

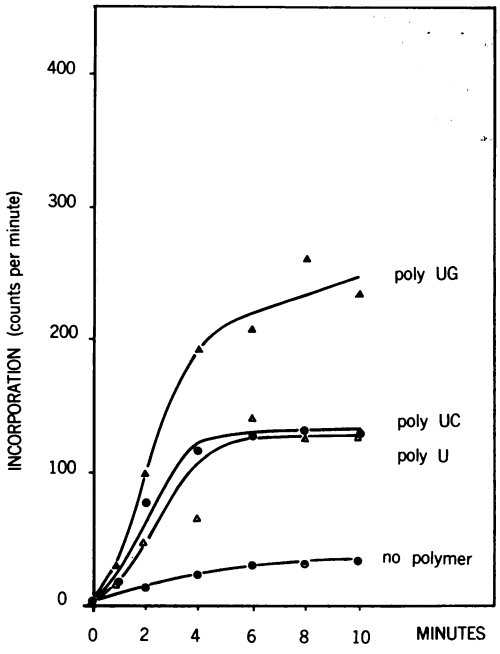

FIG. 4.—Polymer-dependent incorporation of leucine bound to unfractionated *E. coli* sRNA. The reaction mixture and procedure were as in Fig. 2, except that 0.5 mg of C^{14}-leucyl-sRNA, containing 4,300 cpm, was used. The specific activity of the leucine was lower (10 $\mu c/\mu M$) than that used in Figs. 2 and 3.

corresponding to two different coding units. The existence of degeneracy of the second kind, where two different amino acids correspond to the same coding unit, is suggested by the observations that both leucine and phenylalanine respond to poly U.

The present experiments also provide independent proof for the role of sRNA as an adaptor in coding. As shown by Chapeville *et al.*[4] two different amino acids (cysteine and alanine) attached to the same sRNA possess the same coding specificity. In the present experiment, two sRNA's were found to have different coding properties, even though loaded with the same amino acid, showing that the coding specificity resides in the sRNA.

Summary.—*E. coli* contains two leucine-acceptor sRNA's separable by countercurrent distribution. Ribosomal incorporation of leucine attached to one of these is stimulated by poly UC, while leucine attached to the other responds more readily to poly UG. This provides an explanation for the degeneracy observed in coding experiments with leucine. The experiments also confirm that the coding specificity is carried by the sRNA.

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FRACTIONATION OF AMINO ACYL-ACCEPTOR RNA ON A METHYLATED ALBUMIN COLUMN

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The methylated albumin column developed by Mandell and Hershey fractionates DNA by its molecular size, hydrogen bond content, and base composition.[1, 2] It is also capable of separating soluble RNA and ribosomal RNA.[1-5] In this paper, it is shown that the column can be used for fractionating amino acyl-acceptor RNA, and the technique is applied to the amino acyl-acceptor RNA of *Escherichia coli* B. The results show that it is possible to demonstrate heterogeneity in the acceptor RNAs for certain amino acids.

Materials and Methods: Preparation of C^{14} amino acyl-RNA: The method used was similar to that of Berg *et al.*[6] *E. coli* strain B was grown in "C" minimal medium[7] at 37°C with constant shaking. The cells were harvested in the logarithmic phase (A_{650}: 0.3–0.5) and ground with three times their wet weight of alumina (levigated alumina from Norton Abrasives, Worcester, Massachusetts). To the crude extract from a one-liter culture 3 ml of Tris-magnesium buffer (0.01 *M* Tris-HCl buffer, pH 7.3 plus 0.001 *M* $MgCl_2$) was added, and the mixture was centrifuged at 105,000 *g* for 3 hr at 4°C. The upper 2/3 of the supernatant was dialyzed against 500 ml Tris-magnesium buffer plus 2 mg/liter of reduced glutathione at 4°C for 3 hr, changing the outside buffer every half hour.

C^{14}-labeled amino acyl-RNA was prepared by incubating 300 μmoles of Tris-HCl, pH 7.3; 10 μmoles of $MgCl_2$; 6 μmoles of ATP; 3 μmoles of reduced glutathione; 2 ml of the dialyzed 105,000 *g* supernatant equivalent to 1 liter of a logarithmic culture, containing 1 to 1.5 mg of soluble RNA; an appropriate amount of the desired C^{14}-amino acid (see Fig. 2); and, unless otherwise stated, 1 μmole each of the 19 remaining nonradioactive amino acids, in a final volume of approximately 3 ml, for 30 min at 37°C. After 15-min incubation, DNase (10 μg/ml, Worthington Biochemical Corporation, Freehold, N. J.) was added to the mixture, and the incubation was continued for another 15 min. The RNA was then purified by the phenol procedure of Gierer and Schramm.[8] Phenol was removed from the final water layer by extracting with ether, and the RNA preparation was freed from ether by bubbling air through the solution. The RNA was precipitated with 3 volumes of cold ethanol, kept at −15°C for at least 10 min, and centrifuged.